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Arginase in Asthma – Recent Developments in Animal and Human Studies

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Abstract: The enzyme, arginase, converts L-arginine into L-ornithine and urea, and has been implicated in the pathogenesis of lung diseases, related to dysregulation of L-arginine metabolism and remodeling. Allergic asthma is a chronic condition characterized by inflammation, lung remodeling and airways hyperresponsiveness (AHR). Increased expression of arginase may contribute to AHR in asthma by reducing L-arginine bioavailability for the nitric oxide synthase (NOS) isozymes, thus, limiting the production of the endogenous bronchodilator, nitric oxide (NO). Reduction of intracellular L-arginine concentrations as a consequence of augmented arginase expression and activity may also promote NOS uncoupling, resulting in increased formation of peroxynitrite, a powerful oxidant that promotes bronchoconstriction and inflammation. In chronic asthma, increased arginase expression may also contribute to airways remodeling, through increased synthesis of L-ornithine, and hence the production of polyamines and L-proline, which are involved in cell proliferation and collagen deposition, respectively. New drugs targeting the arginase pathway could have therapeutic benefits in asthma. This review focuses on recent developments in our understanding of the role of arginase in AHR, inflammation and remodeling, highlighting studies that advance our knowledge of L-arginine dysregulation in human asthma and animal studies that explore the therapeutic potential of arginase inhibition.

Keywords: Nitric oxide, L-arginine, airways remodeling, inflammation, airway hyperresponsiveness, polyamines, fibrosis, proline, peroxynitrite.

INTRODUCTION

The metabolism of the semi-essential amino acid L-arginine has been of great interest since the Nobel Prize winning research programs of Furchgott, Ignarro and Murad, which led to the discovery of nitric oxide (NO) as a bioactive molecule. For many years, the catabolism of L-arginine to NO and L-citrulline was considered the most important biologically active pathway. In recent years, however, metabolism of L-arginine to urea and ornithine *via* the arginase family of isozymes has emerged as an important pathway in numerous physiological and pathophysiological conditions, including lung disease.

SUMMARY OF REVIEWS TO DATE

Reflecting the increased interest in arginase, there have been several recent reviews describing the role in lung disease and immunology. Maarsingh *et al.* recently described the therapeutic potential of arginase inhibition in asthma [1]. A review by Meurs *et al.* focused on the airways hyperresponsiveness of asthma both *in vitro* and *in vivo* in animal models of variable and chronic AHR [2]. The role of arginase in the regulation of airway smooth muscle tone by the nervous system was also described, along with the role of arginase in the chronic structural changes and remodeling of airway smooth muscle in AHR [2]. Maarsingh *et al.* reviewed the current knowledge of arginase induction in a variety of pulmonary diseases including asthma, COPD, cystic fibrosis, pulmonary hypertension, and remodeling and fibrotic disorders [3]. The unifying role of arginase in these diseases of the lung was the regulation of the synthesis of NO, polyamines and L-proline [3], contributing to smooth muscle reactivity, tissue hypertrophy/hyperplasia and collagen deposition, respectively. In another review from 2008, Maarsingh *et al.* reviewed the importance of altered L-arginine metabolism in AHR, inflammation and remodeling in allergic asthma, focusing on the three mechanisms that regulate L-arginine bioavailability within the cell: uptake, catabolism and recycling from L-citrulline [4]. In 2007, Peranzoni *et al.* reviewed the potential roles of L-arginine metabolism in the early and late immune response to inflammatory insults and in the pathology of a variety of neoplastic and autoimmune diseases [5]. Mori reviewed the regulation of apoptosis by the balance of nitric oxide synthesis through arginase and L-arginine recycling through L-citrulline [6]. Morris also reviewed the current state of knowledge about L-arginine metabolism in 2007, including transport into the cell and mitochondria and its

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interconversion from and into different amino acids as well as the multiple products of different pathways of L-arginine catabolism [7]. The interested reader is directed to these comprehensive reviews. The aim of the current review is to unify the recent advances in our knowledge of arginase in asthma, specifically focusing on the *in vivo* inhibitory studies, and reports that translate L-arginine metabolism *via* arginase to human asthma.

NITRIC OXIDE HOMEOSTASIS AND AIRWAY FUNCTION

NO is synthesized from L-arginine in a 2-step reaction with N^{ω}-hydroxy-L-arginine (NOHA) as an intermediate by a family of NO-synthases that utilize oxygen and NAPDH as cosubstrates and yield L-citrulline as a coproduct [8,9]. Three NOS isoforms that are expressed from three distinct gene products have been identified: neuronal NOS (nNOS or NOS I) and endothelial NOS (eNOS or NOS III), which are constitutively expressed, and inducible NOS (iNOS or NOS II). In the airways, the constitutive NOS (cNOS) isozymes are mainly expressed in airway epithelium (NOS1 and NOS3), inhibitory nonadrenergic noncholinergic (iNANC) neurons (NOS1), and in endothelial cells from the airway vasculature (NOS3). cNOS isoforms produce relatively low levels of NO in response to increases in intracellular Ca^{2+}, evoked by membrane depolarization or by contractile agonists [10]. The activity of NOS2, which produces large amounts of NO, is regulated at the expressional level, e.g. by proinflammatory cytokines during airway inflammation [10-12].

NO is an important endogenous bronchodilator, inducing airway smooth muscle relaxation via mechanisms that are both dependent and independent of cGMP, generated through activation of soluble guanylyl cyclase by NO, collectively leading to decreased cytosolic Ca^{2+}-concentrations, decreased Ca^{2+}-sensitivity, and membrane hyperpolarization of the smooth muscle cell [10]. The bioavailability of L-arginine for the NOS isozymes determines the formation of NO in the airways, as illustrated by the finding that in healthy subjects the levels of exhaled NO are increased by oral or inhalational administration of L-arginine [13-15]. In guinea pig airway preparations *in vitro*, L-arginine diminishes airway constriction by methacholine [16], and increases iNANC nerve-mediated smooth muscle relaxation [17], as a consequence of increased formation of cNOS-derived NO.

In many cells, the availability of L-arginine for the NOS isozymes depends on L-arginine uptake by the cell through specific cationic amino acid transporters (CATs), recycling of L-citrulline to L-arginine, and on the activity of arginase which competes with NOS for the common substrate [4]. Uptake of L-arginine via the Na^{+}-independent CATs of the y^{+}- system (Fig. **1**), the major transcellular L-arginine transporter [18], can be inhibited by other cationic amino acids such as L-ornithine and L-lysine, as well as by polycations, including major basic protein (MBP) from eosinophils [19]. Thus, MBP and its analogue poly-L-arginine attenuates L-arginine uptake and NO-synthesis in alveolar macrophages and tracheal epithelial cells [19], and increases airway responsiveness of rats and guinea pigs to methacholine, *in vivo* and *in vitro* [20,21] by inducing a deficiency of NO [21]. The L-arginine availability may also be regulated by recycling of the NOS-product L-citrulline, through the L-citrulline/L-arginine cycle [22,23]. This cycle consists of the enzymes L-argininosuccinate synthase and L-argininosuccinate-lyase (ASS and ASL, respectively), which, in various tissues, are colocalized and coinduced with NOS isozymes [23-27]. Although recycling of L-citrulline does not play a role in iNANC NO-mediated relaxations of human and guinea pig airway smooth muscle under basal

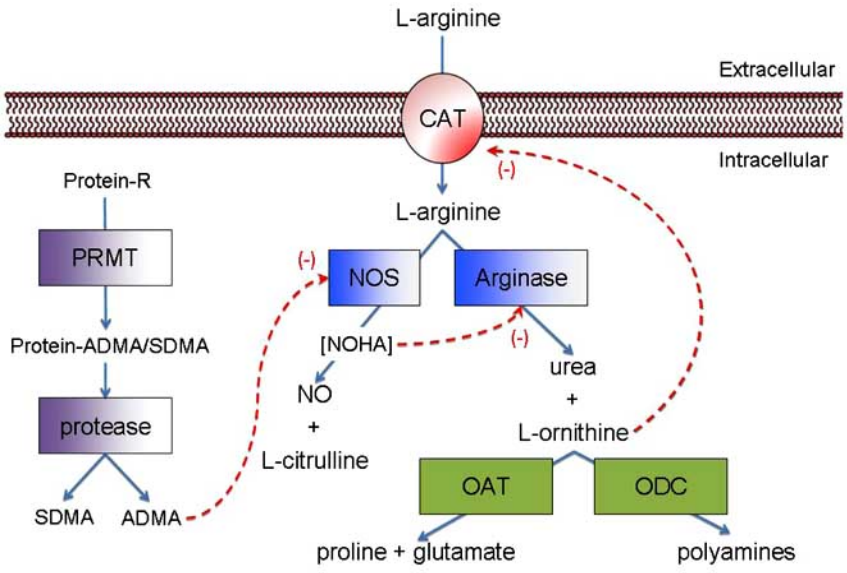

Fig. (1). Interactions between L-arginine metabolic pathways.

ADMA, asymmetric (N^G,N^G) dimethyl arginine; CAT, cationic amino acid transporters; NO, nitric oxide; NOHA, N^{ω}-hydroxy-L-arginine; NOS, nitric oxide synthase; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; PRMT, protein arginine methyl transferase; SDMA, symmetric (N^G,N^G) dimethyl arginine.

conditions, the inhibitory effect of the NOS-inhibitor N^{ω}-nitro-L-arginine was reversed by L-citrulline [28,29], which was prevented by ASS and ASL inhibitors, indicating that the L-citrulline/L-arginine cycle is effective under conditions of low L-arginine utilization by NOS1 [29]. For a detailed review on the role of L-arginine transport and L-citrulline recycling in regulating airway function in health and disease see: [4].

The third and most studied mechanism that regulates the L-arginine availability to NOS is substrate competition by arginase, which converts the amino acid into L-ornithine and urea [4,30], and will be the focus of the current review. Although the K_m of L-arginine for arginase is up to 1000-fold higher than for NOS, substrate competition can occur, because the V_{max} is also 1000-fold higher [30-32]. Two isozymes, arginase 1 and arginase 2, encoded by different genes, have been identified [30,33,34]. Arginase 1 is the major isozyme in the liver and is involved in the urea cycle, whereas arginase 2 is predominantly expressed extrahepatically [30,35,36]. In the airways, both isozymes are constitutively present in structural cells, including the epithelium, endothelium, (myo)fibroblasts and airway smooth muscle cells [37-40], as well as in inflammatory cells, such as alveolar macrophages and neutrophils [36,37,41]. It is becoming increasingly appreciated that the cellular and sub-cellular localization of these two isozyme families likely contributes to their potential roles in homeostasis and disease pathology (this will be discussed later in this review).

The significance of arginase in regulating airway function is clearly demonstrated by the effects of the potent and specific arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) on epithelial-derived and nerve-derived NO-generation, even in non-diseased airways. Thus, nor-NOHA attenuated methacholine-induced constriction of intact perfused guinea pig tracheae as well as iNANC-mediated relaxations of isolated tracheal smooth muscle preparations [17,42]. These effects of nor-NOHA were quantitatively similar to those of L-arginine, indicating that substrate competition between NOS and arginase is indeed involved [16,17,42].

However, in the non-inflamed mouse airways, inhalation of arginase-specific inhibitors (i.e., BEC [43] and nor-NOHA) did not affect basal central airways or total lung resistance, whereas the nonspecific arginase inhibitor NOHA, which also acts as substrate for the NOS isozymes and thus increase NO levels directly, reduced baseline tone (North *et al.,* unpublished observation). The ineffectiveness of specific arginase inhibitors is is likely a consequence of the low-levels of arginases expressed in normal, non-diseased lungs [43]. Furthermore, in unrestrained guinea pigs, permanently instrumented with a pulmonary balloon catheter for on-line measurement of pleural pressure, inhalation of the potent and isozyme-nonselective arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH) or L-arginine did not decrease the basal responsiveness of the airways to histamine, measured as a 100% increase of pleural pressure, either [44]. Thus, it would appear that under normal homeostatic conditions in the airways *in vivo*, arginase and NOS do not compete significantly for L-arginine as substrate.

In alveolar macrophages, arginase was found to regulate NO-production by NOS, and *vice versa*, Thus, NOS was able to inhibit arginase activity through NOHA, the intermediate product in NO synthesis [45]. The bioavailability of L-arginine to NOS could also be regulated by the arginase product L-ornithine, which competitively inhibits the uptake of L-arginine via specific CATs of the y^+-system [46-48]. Consistent with this finding, incubation with L-ornithine increased airway responsiveness in perfused guinea pig tracheal preparations by inducing a deficiency of cNOS-derived NO [49]. Although L-ornithine also competitively inhibits arginase activity [32,49,50], the increase in airway responsiveness shows that the inhibitory effect on L-arginine uptake is larger than that on arginase activity [49]. Thus, mutual interactions between NOS and arginase regulate L-arginine homeostasis and NO-synthesis in the airways (Fig. **1**) and changes in this balance may underlie the pathophysiology of obstructive airways diseases, including asthma.

ARGINASE IN ASTHMA

Allergic asthma is a chronic inflammatory disorder of the airways that is characterized by reversible airways obstruction. Characteristic features of the disease are allergen-induced, IgE-mediated early (EAR) and late (LAR) asthmatic reactions, infiltration of inflammatory cells - particularly eosinophils and Th2 lymphocytes - into the airways, and inflammation-induced acute and transient airway hyperresponsiveness (AHR) that is present both after the EAR and LAR [51,52]. Chronic airway inflammation may induce airway remodeling, characterized by structural changes in the airway wall, including subepithelial fibrosis and increased airway smooth muscle mass [51,53,54]. Airway remodeling may contribute to a progressive decline in lung function, as well as to the development of persistent AHR [51,52]. The mechanisms underlying AHR are only partially understood. However, recent evidence suggests that alterations in L-arginine metabolism induced by arginase may be importantly involved in both acute and chronic AHR, via altered NO metabolism and synthesis of polyamines and L-proline, respectively [2,4,55].

Role of Altered NO Metabolism in Acute Allergen-Induced AHR

In asthmatic patients, the production of NO in the airways is considerably increased due to marked upregulation of NOS2 by pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and IL-1β [10]. The increased NO production by NOS2 is associated with increased concentrations of NO in the exhaled air [14]. In experimental asthma, NOS2 is induced during the allergen-induced LAR, similarly leading to enhanced levels of NO in the exhaled air [56,57]. Significant correlations between exhaled NO, NOS2 expression in airway epithelial and inflammatory cells, airway eosinophilia and AHR have been observed in asthmatics, whereas all are reduced after glucocorticosteroid treatment [58-60]. Therefore, the NO concentration in exhaled air is considered as a sensitive marker of airway inflammation in asthma [61].

However, many studies have indicated that airway inflammation is not primarily caused by high concentrations of NOS2-derived NO by itself, but rather by increased

formation of peroxynitrite ($ONOO^-$), a highly reactive oxidant synthesized by the reaction of NO with superoxide anions (O_2^-) in the inflamed airways [58,62]. Thus, whereas NO causes bronchodilation and has anti-inflammatory actions by inhibiting the activation of NF-κB [10,11,63-65], peroxynitrite has shown to induce pro-inflammatory effects associated with epithelial damage, eosinophil activation, MUC5AC expression, and vascular hyperpermeability [66-69]. In addition, peroxynitrite promotes airway smooth muscle contraction [12,70]. Accordingly, increased nitrotyrosine immunostaining, indicative of protein tyrosine nitration by peroxynitrite, has been observed in airway epithelial and inflammatory cells of bronchial biopsies from asthmatic patients as well as in airways of allergen-challenged guinea pigs [58,67]. Moreover, increased levels of exhaled NO correlate with increased nitrotyrosine concentrations in exhaled breath condensate of asthmatic patients [71], while nitrotyrosine staining in bronchial biopsies of these patients was shown to correlate with NOS2 expression, eosinophilic airway inflammation and AHR [58]. In line with these observations, animal studies have indicated that increased formation of peroxynitrite may importantly contribute to the development of allergen-induced AHR after the LAR in acute and chronic asthma [12,62,66,70,72].

Remarkably, in transgenic mice overexpressing NOS2 in the airways, increased levels of exhaled NO were associated with reduced airway resistance and airway *hypo*responsiveness, with no signs of airway inflammation and increased nitrotyrosine staining, indicating that increased production of NO *per se* is not detrimental, but rather protective [73]. Moreover, allergen-induced airway inflammation and AHR were more pronounced in NOS2 knock-out mice than in wild type animals [74], although unchanged AHR [75] and inflammation [75,76] as well as reduced inflammation [77,78] have also been reported (for a comprehensive review on the role of NOS isoforms in asthma, see Mathrani *et al* [79]).

Despite the increased concentration of NO in exhaled air of asthmatics, various studies in animal models and in asthmatic patients have now indicated that acute allergen-induced AHR is paradoxically due to a deficiency of bronchodilating and anti-inflammatory NO in the airway wall, which may be caused by a reduced L-arginine availability to both cNOS and NOS2 [3,4,55,80]. The first evidence for a role of reduced production of cNOS-derived NO in allergen-induced AHR came from studies in guinea pig models of acute allergic asthma. In these models, NOS2 is not detected before the LAR [57], making it possible to investigate the distinct role of cNOS-derived NO in the EAR and the AHR immediately after the EAR. In an initial study, it was demonstrated that NO in the exhaled air drops below baseline during the allergen-induced EAR, which might contribute to the obstructive reaction as well as to the ensuing AHR [81]. Indeed, both *in vivo* and *ex vivo* studies using a guinea pig model of acute allergic asthma indicated that a deficiency of contractile agonist-induced cNOS-derived NO is an important determinant of the AHR after the EAR [82-84]. A deficiency of cNOS activity and endogenous bronchodilating NO causing AHR was also demonstrated after repeated allergen challenges of sensitized guinea pigs [85]. Interestingly, a deficiency of iNANC nerve-derived NO was also found after single or repeated allergen challenges [86,87]. Ricciardolo and coworkers importantly demonstrated that a reduction in cNOS-derived NO also contributed to the AHR in patients with severe asthma treated with glucocorticosteroids, that inhibit the expression of NOS2 [88]. Moreover, as in guinea pigs, the deficiency of cNOS-derived NO could be induced by allergen challenge [89]. Interestingly, reduced cNOS-(presumably iNANC nerve)- derived NO could also be involved in attenuated deep inspiration-induced bronchoprotection in asthmatic patients [90-92].

Animal studies further indicated that reduced L-arginine bioavailability to cNOS importantly accounts for the NO deficiency after the allergen-induced EAR (for review see [4]). Thus, exogenous L-arginine reduced the AHR to methacholine in perfused tracheal preparations of allergen-challenged guinea pigs *ex vivo* [16], and increased iNANC nerve-mediated airway smooth muscle relaxation in tracheal open-ring preparations from these animals [86]. Moreover, it was recently demonstrated that inhalation of L-arginine attenuates the allergen-induced AHR to histamine after the EAR in guinea pigs *in vivo* [44].

Since it has been shown that (particularly NOS3-derived) NO inhibits airway inflammation by suppression of NF-κB activity, thereby inhibiting the expression of pro-inflammatory cytokines as well as of NOS2 [63-65,93], the deficiency of cNOS-derived NO after the EAR could also contribute to the inflammatory response during the LAR. Accordingly, in asthmatic patients significant correlations were found between allergen-induced AHR after the LAR and reduced NOS3 expression as well as increased NOS2 expression in bronchial biopsies [89]. Moreover, allergen-induced airway inflammation was markedly reduced in NOS3-overexpressing mice as compared with wild-types [93,94]. Furthermore, in the acute ovalbumin sensitization and challenge model in mice, pulmonary NOS2 expression is up-regulated and NOS3 expression is down-regulated [43], likely further contributing to peribronchiolar inflammation in this model.

The enhanced production of peroxynitrite observed after the LAR could be caused by reduced L-arginine bioavailability to NOS2. Thus, studies in macrophages have indicated that under conditions of low L-arginine availability NOS2 not only produces NO by its oxygenase moiety, but also synthesizes superoxide anions by the reductase moiety, leading to extremely efficient formation of peroxynitrite [95]. Increasing the L-arginine concentration in these cells stimulates NO production, while reducing the production of superoxide anions, and hence peroxynitrite [96]. The potential importance of this mechanism in the AHR after the LAR was recently indicated by observations in a guinea pig model of asthma, demonstrating that administration of exogenous L-arginine inhibits the allergen-induced AHR after the LAR both *ex vivo* and *in vivo* [44,97].

Since the bioavailability of L-arginine to NOS isozymes is regulated by arginase, increased consumption of the amino acid by arginase could explain the L-arginine limitation and subsequent AHR after the EAR and LAR in allergic asthma. In support of this hypothesis, the arginase activity in tracheal homogenates obtained from ovalbumin-challenged guinea pigs after the EAR was increased almost 4-fold compared to controls [98], whereas a 2-fold increase was observed after

the LAR [97]. Ovalbumin also strongly increased lung arginase activity in mouse [43,99,100] and rat [101] models of acute allergic asthma. The arginase activity in mouse lung was also increased after challenge with *Dermatophagoides farinae* [102], *Aspergillus fumigatus* [99] and trimilletic anhydride [100], suggesting that arginase is a key mediator in the response to allergic and occupational stimuli.

A number of studies using different mouse models of allergic asthma have investigated mRNA or protein expression of arginase 1 and 2 after allergen challenge (for review see [3]). Both isoforms are induced in lungs of mice challenged with either ovalbumin [43,99], *D. farinae* [102], *A. fumigatus* [99], trimilletic anhydride [100] or *S. mansoni* eggs [103], the induction of arginase 1 being the most prominent. Microarray analysis of gene expression in mouse lung after challenge with either ovalbumin or *A. fumigatus* showed that among the 291 genes that were commonly induced by these allergens, the genes encoding for arginase 1 and 2 were among the most predominantly overexpressed [99]. Th2 cytokines, which are elevated in allergic asthma, seem to play an important role in the induction of arginase 1 [99,103-105] and – to a lesser extinct – arginase 2 [99]. In addition, arginase 1 was among the 26 commonly expressed transcripts that were (strongly) increased in lung inflammation in 5 different mouse models of Th2 cytokine-mediated inflammation [106]. The cytokine-induced expression of arginase 1 is under important control of the transcription factors STAT6 and CCAAT-enhancer binding protein [99,104,107-109], whereas the induction of arginase 2 is largely STAT6 independent [99].

Role of Arginase in Allergen-Induced NO Deficiency and Acute AHR *Ex vivo*

To study whether increased arginase activity indeed contributes to the reduced bioavailability of L-arginine and subsequent NO deficiency and AHR after the EAR, the effect of the specific arginase inhibitor nor-NOHA was studied in perfused tracheal preparations obtained from allergen-challenged guinea pigs [98]. Interestingly, the allergen-induced AHR after the EAR was completely inhibited by nor-NOHA to the level of normoreponsive unchallenged controls [98]. The effect of nor-NOHA was completely prevented by coincubation with the NOS inhibitor L-NAME, clearly demonstrating that arginase inhibition reduces the AHR by restoring the production of cNOS-derived NO [98]. Treatment with nor-NOHA also restored the impaired iNANC-nerve mediated NO production and airway smooth muscle relaxation after the EAR [86]. The effects of nor-NOHA were quantitively similar to the effect of treatment with L-arginine [86,98]. The importance of increased arginase in limiting the L-arginine bioavailability and NO production is supported by the observation that levels of L-arginine and L-citrulline are reduced in the airways of allergen-challenged mice, whereas the arginase activity is increased [110]. In conclusion, increased arginase activity underlies the deficiency of neuronal and nonneuronal NO and subsequent AHR after the EAR by limiting the substrate availability to cNOS isoforms. Of note, since L-arginine transport via the y^+-system is inhibited by L-ornithine [46-48] leading to a deficiency of NO and increased airway responsiveness [49], increased formation of L-ornithine by arginase could also contribute to the L-arginine limitation and NO deficiency in allergic asthma.

As indicated above, the AHR after the LAR is caused by increased formation of peroxynitrite [12], due to reduced bioavailability of L-arginine for NOS2, which causes uncoupling of this enzyme [95,97]. Since the arginase activity in the airways of these animals is increased [97], increased consumption of L-arginine by arginase could by involved in this process. Indeed, inhibition of arginase reduced the AHR by restoring the production of bronchodilating NO, presumably by preventing the uncoupling of NOS2 [97]. These findings clearly indicate that the AHR after the LAR is caused by increased formation of peroxynitrite and not by an excess of NO. Increased arginase activity similarly underlies the peroxynitrite-mediated AHR in a guinea pig model of chronic asthma [72]. The importance of arginase in uncoupling of NOS2 and subsequent peroxynitrite formation is further supported by the observation that increased expression of arginase and NOS2 are colocalized with increased nitrotyrosine staining in lungs of *D. farinae*-challenged mice [102].

Collectively, these functional *ex vivo* studies demonstrate that increased arginase activity contributes importantly to allergen-induced AHR after both the EAR and LAR by limiting the bioavailability of L-arginine to the NOS isozymes, leading to a deficiency of bronchodilating NO and increased formation of peroxynitrite.

In vivo Treatment with Arginase Inhibitors

The effectiveness of arginase inhibition in reducing *in vivo* AHR in allergic asthma has been recently studied in different animal models using a number of inhibitors (Table **1**). The first report on the effect of arginase inhibitors on AHR *in vivo* was in a guinea pig model of acute allergic asthma, measuring airway responsiveness to inhaled histamine in permanently instrumented, freely moving animals. In these animals, treatment via inhalation with the specific arginase inhibitor ABH at 5.5 and 23.5 h after a single allergen challenge acutely reversed the AHR after the EAR and the LAR [44]. Using the same animal model of asthma, the AHR after the EAR was also acutely reduced after treatment with inhaled nor-NOHA (**Table 1**; Maarsingh *et al*., unpublished observation). Similar to the finding in guinea pigs, inhalation of nor-NOHA acutely reversed the allergen-induced AHR of the central airways to inhaled methacholine in an acute murine model of allergic airways inflammation (**Table 1**; North *et al*., unpublished observation). Moreover, in these mice inhalation of the specific arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC) normalized the allergen-induced AHR of the central, but not the peripheral, airways (**Table 1**) [43]. This finding contradicts a previous observation in mice, showing that oropharyngeal aspiration of BEC 2 h after the last of three ovalbumin challenges did not affect AHR of the central airways 46 h later [111]. In these animals, BEC did not reduce – but rather accelerated - the peak-response of the peripheral airways to methacholine [111]. An explanation for the discrepancies is currently not at hand. In addition to the specific arginase inhibitors ABH, BEC and nor-NOHA, inhaled treatment with the non-specific arginase inhibitor, NOHA, normalized the AHR to methacholine in a mouse

Table 1. Effectiveness of Arginase Inhibitors in Reducing (Allergen-Induced) AHR *In Vivo* in Various Animal Models of Asthma

Inhibitor	Species	Route	Dose	Inhibition of AHR	Asthma Model	References
ABH	Guinea pig	Inhalation				
		- acute[a]	25 mM[i]	60% (EAR);	Acute OVA	[44]
		- protective[b]	25 mM[i]	61% (LAR)	Acute OVA	[44]
				85% (EAR);		
				full (LAR)		
BEC	Mouse	Inhalation				
		- acute[d]	40 μg/g[k]	Full	Acute OVA	[43]
		- acute[e]	40 μg/g[k]	Full	Chronic OVA	[43]
		Oropharyngal				
		aspiration[c]	0.3 mM[j]	No inhibition	Acute OVA	[111]
Nor-NOHA	Guinea pig	Inhalation				
		- acute[f]	5 mM[i]	48% (EAR)	Acute OVA	[*]
	Mouse	Inhalation				
		- acute[d]	40 μg/g[k]	43%	Acute OVA	[**]
	Mouse	Intraperitoneal				
		- protective[g]	100 μg[l]	32%	Acute OVA	[74]
NOHA	Mouse	Inhalation				
		- acute[d]	40 μg/g[k]	Full	Acute OVA	[**]
Arginase 1	Mouse	Intratracheal				
RNAi		instillation[h]	25 μg[m]	Full	Acute IL-13	[104]

[a]Assesment of AHR at 5 h (EAR) and 23 (LAR) after single allergen challenge, treatment 15 min before reassessment of AHR at 6 h and 24 h;
[b]treatment 0.5 h before and 8 h after single allergen challenge, AHR assessed at 6 h (EAR) and 24 h (LAR);
[c]treatment 2 h after allergen challenge, AHR assessed at 48 h;
[d]treatment 15 min before assessment of AHR at 24 h after the last of 6 daily allergen challenges;
[e]treatment 15 min before assessment of AHR at 24 h after the last of 12 allergen challenges.
[f]Assesment of AHR at 5 h (EAR) after single allergen challenge, treatment 15 min before reassessment of AHR at 6 h;
[g]treatment 0.5 h before each of 6 allergen challenges, AHR assessed 1-3 h after the last challenge;
[h]treatment 27 h and 24 h before IL-13 instillation, AHR assessed 12 h after IL-13 instillation;
[i]nebulizer concentration in an 8 L cage;
[j]total volume of 40 μl, equals ~3 μg BEC;
[k]body weight, equals ~800 μg BEC;
[l] in 100 μl PBS;
[m] 25 μg of plasmid in 50 μl ExGen500 and 5% w/v glucose;
[*], Maarsingh *et al*, unpublished observation;
[**] North *et al*, unpublished observation.
Abbreviations: ABH, 2(S)-aminoboronohexanoic acid; AHR, airway hyperresponsiveness; BEC, S-(2-boronoethyl)-L-cysteine; EAR, early asthmatic reaction; LAR, late asthmatic reaction; OVA, ovalbumin; NOHA, N^{ω}-hydroxy-L-arginine; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine.

model of allergic asthma (**Table 1**; North *et al*., unpublished observation). However, NOHA is also substrate for NOS. Therefore, the effect of NOHA may at least partially result from a direct stimulation of NO synthesis, in addition to increasing the L-arginine availability to NOS by inhibiting arginase.

AHR to inhaled methacholine persists in a mouse model of chronic allergic asthma, in which the animals were challenged every other week for 2 consecutive days for a period of 12 weeks [43]. Treatment with inhaled BEC 24 h after the last of 12 allergen challenges completely normalized the AHR of the central as well as the peripheral airways in these mice (**Table 1**), clearly indicating that increased arginase activity also contributes to the AHR *in vivo* in chronic asthma [43]. Furthermore, in the chronic asthma model the isozyme responsible for the AHR appears to be arginase 1, since the expression of arginase 2 is not increased in this model [43].

Distinct from the aforementioned treatment-based protocols, the protective effect of arginase inhibitors on allergen-induced AHR in asthma has also been studied in both guinea pigs and mice. Remarkably, the sensitivity of the airways to the inhaled allergen of sensitized guinea pigs was largely reduced after treatment with inhaled ABH 0.5 h prior to allergen challenge as compared to pretreatment with saline [44]. This anti-allergic effect of the arginase inhibitor may involve increased formation of NO, which is known to inhibit mast cell activation, as well as a number of mast cell-

mediated inflammatory processes [112]. In addition, NO inhibits the allergen-induced mast cell mediator release from guinea pig lung parenchymal tissue [113]. When saline- and ABH-pretreated animals were challenged to a similar bronchial obstruction, thus requiring a *higher* allergen dose in the ABH-treated animals, the AHR after the EAR as well as after the LAR was partially reduced in the ABH-treated animals [44]. However, almost full inhibition of the AHR after the EAR and full inhibition of the AHR after the LAR were obtained, when the ABH-treated guinea pigs were challenged with the *same* allergen dose that induced airway obstruction in saline-treated animals (**Table 1**) [44]. The initial allergen-induced bronchial obstruction and the magnitudes of the early and late asthmatic reactions were also greatly reduced by pretreatment with ABH [44]. The protective effect of arginase inhibitors on the development of allergen-induced AHR was confirmed in another mouse model of asthma, showing that treatment with intraperitoneally applied nor-NOHA 0.5 h before each of 6 allergen challenges, over the course of two weeks, significantly reduced the AHR to methacholine assessed 1-3 h after the last allergen challenge (**Table 1**) [74]. In addition, treatment with interference RNA against arginase 1 prevented the IL-13-induced upregulation of arginase 1 in mouse lung as well as the AHR induced by this cytokine [104]. In this mouse model, increased arginase 1 expression correlated temporally with the development, persistence, and resolution of IL-13-induced AHR [104]. All arginase inhibitors studied in the *in vivo* models are subtype nonselective and therefore the contribution of the two arginase isoforms to the development of allergen-induced AHR in these models is not known. However, whereas both isoforms were increased in the acute mouse model, only arginase 1 was upregulated in the chronic mouse model [43].

In conclusion, targeting increased arginase activity using (inhaled) arginase inhibitors may be a novel therapeutic strategy in allergic asthma, since these inhibitors acutely reduce allergen-induced AHR, have anti-allergic properties and protect against the development of allergen-induced AHR and bronchial obstructive reactions.

Role of Arginase in Human Asthma

There is increasing evidence that arginase is also important in the pathophysiology of human asthma [114]. Quite remarkably, as early as in 1980 enhanced arginase activity was found in sputum from asthmatic patients, which at that time was ascribed to disturbed liver function due to hypoxia [115]. It took, however, more than 20 years to appreciate the potential importance of this finding [55,98,99,114]. Increased arginase expression and/or activity have now also been demonstrated in airway and lung tissue, BAL cells and serum from asthmatic patients [39,43,99,114,116].

Increased protein and mRNA expression of arginase 1 in asthmatic patients was first described by Zimmerman *et al*. [99]. Northern blot analysis revealed increased mRNA expression of arginase 1 in inflammatory cells and airway epithelium in bronchial biopsies of these patients, while increased protein expression of the enzyme was observed in inflammatory cells, presumably macrophages, in the BAL. Accordingly, the recent study by North *et al.* [43] demonstrated increased arginase activity and protein expression of arginase 1, but not arginase 2, in lung tissues from asthmatic patients. Furthermore, immunohistochemical studies have indicated augmented arginase 1 protein expression in the airway epithelium and airway smooth muscle layer of smoking asthmatics compared to non-smoking patients, which might be induced by nicotine [39].

Remarkably, in asthmatic patients experiencing an exacerbation, arginase activity in serum was considerably increased, whereas L-arginine levels in the plasma were strikingly reduced [116]. Moreover, improvement of asthma symptoms in some of these patients was associated with a decline in arginase activity and increased L-arginine concentrations [116]. These findings indicate that changes in arginase expression are not solely confined to the airways and that reduced levels of circulating L-arginine could contribute to NO deficiency in the airways. In support, in patients with severe asthma serum arginase activity was inversely correlated with FEV_1 and FEV_1/FVC, whereas a positive correlation was found between L-arginine bioavailability and lung function [114]. Of interest is the recent finding that human neutrophils contain high levels of arginase in azurophilic granules [41], as it is known that infiltration and activation of these cells in the airways is particularly enhanced in severe asthma.

In addition to the changes in expression and activity of arginase described above, single nucleotide polymorphisms (SNPs) in the arginase 1 and 2 genes have recently also been associated with allergic asthma. Thus, a significant association between SNPs in arginase 2 and increased risk of childhood asthma has been observed, whereas in the same population of children SNPs in both arginase 1 and arginase 2 were associated with atopy [117]. Quite remarkably, pharmacogenetic screening of 844 SNPs in 111 candidate genes for association with inhaled β_2-agonist-induced bronchodilation recently identified arginase 1 as a potential β_2-agonist response gene [118].

ARGINASE IN INFLAMMATION

Persistent inflammation plays a defining role in asthma, and one of the central goals of current asthma therapy is to minimize this process [119,120]. Inflammation and expression of arginase has also been demonstrated in all commonly-studied animal models of allergic inflammation, including rat, guinea pig, and mouse, as well as in human asthma [39,43,86,97-99,101,110]. While arginase expression has been found to be upregulated in epithelia, smooth muscle and the peribronchiolar region [39,43,99,102], this section will focus on new developments in our understanding of arginase expression and function in immune cells.

In 1995, Corraliza and Modolell reported the induction of arginase in murine macrophages by Th2 cytokines [121, 122]. Further studies have revealed upregulation of arginase1 by IL-4 and IL-13, through a STAT6-dependent enhancer-mediated mechanism [109,123]. Increased arginase 1 mRNA expression was demonstrated in two independent murine models of allergic airways inflammation by Zimmermann *et al.*, localized to macrophages and areas of peribronchiolar inflammation [99]. Arginase-positive inflammatory cells were also demonstrated in the mucosa of human asthmatic bronchial biopsy and bronchoalveolar lavage samples [99].

Following these seminal findings, arginase has been found to be highly expressed in alternatively-activated macrophages [124,125], and to play an important role in allergic inflammation and asthma [43,100-102,104,110] . Relatively little information is available regarding the expression and function of arginase in human inflammatory cells, compared to murine models. This section will provide an overview of arginase in immune cells, as it is reviewed comprehensively elsewhere in this supplement.

Munder *et al*. [41] reported the first comparative examination of arginase expression in human and murine immune cells and showed that in contrast to the highly-inducible expression of arginase that occurs in murine macrophages and dendritic cells, human polymorphonuclear leukocytes and eosinophils express arginase 1 constitutively; with no significant inducibility in any of the human immune cell populations. Arginase is sequestered within azurophilic granules in these cell types, and exocytosed after activation [41,126]. As such, the release of arginase 1 by PMNs contributes towards the antimicrobial/fungicidal activity [41], controls L-arginine concentrations in the local microenvironment, and supresses the activation of T-lymphocytes [127] and natural killer cells [128]. The subcellular localization of arginase in PMNs has been challenged by Jacobsen *et al*., who described that arginase 1 is localized to gelatinase granules of human PMNs, as opposed to the earlier-stage azurophilic granules [129]. Since considerable heterogeneity in infiltrating cell types can exist between asthmatic individuals, and since asthmatic subtypes with severe disease can exhibit predominantly neutrophilic inflammation [130-133], arginase induction within this cell population could likely contribute to arginine dysregulation in human asthma.

Murine macrophages are capable of inducing expression of arginase 1 in response to various stimuli, including Th2 cytokines and transforming growth factor (TGF) [134]. Furthermore, Erdely *et al.* also demonstrated that arginase 1 could be induced by IL-4 in combination with pharmacologic tools that increase cAMP levels (i.e., forskolin, which activates adenylate cyclase, and phosphodiesterase inhibitors) in both human alveolar macrophages and murine RAW264.7 cells [134]. Thus, the potential for arginase to play a role in arginine balance/imbalance in macrophages is likely dependent upon the cell populations studied and the conditions used for induction. Understanding the complex control of arginase expression within these cells will be important to advance research in allergic asthma.

Arginase and Alternatively-Activated Macrophages

The primary function of classically-activated macrophages is the cytotoxic killing of microbes through the production of reactive oxygen and nitrogen species, including high levels of NO produced by NOS2. Classical activation of macrophages depends on cytokines derived from activated $CD4^+$ T helper 1 cells (Th1), particularly IFN-γ [125]. Alternative activation of macrophages was proposed in the early 1990's, as a phenotype induced by the Th2 cytokine IL-4 and characterized by suppression of IL-1 synthesis and expression of the macrophage mannose receptor [135,136]. Alternative-activation is characterized by the expression of several signature proteins, including arginase 1, chitinase-like molecules (Ym1, Ym2 and acidic mammalian chitinase; AMCase), and resistin-like molecule (RELMα, also known as FIZZ1) [124]. The cells responsible for producing IL-4 and initiating the alternative activation of macrophages include $CD4^+$ Th2 cells, $CD8^+$ T cells, NK cells, basophils, mast cells, and eosinophils, indicating that alternative activation can be elicited by either the innate or the acquired immune system [125]. Alternative-activation and expression of arginase 1 has been described in macrophages in a variety of diseases, including interstitial pulmonary fibrosis [137]. Arginase likely plays a detrimental role by limiting substrate for NOS2 in diseases in which a Th1 response is required. However, arginase expressed in alternatively-activated macrophages likely plays a role in T cell regulation, and the shift of L-arginine metabolism from NO production to the synthesis of polyamines and proline is likely important for the resolution of inflammation and wound healing [125,138,139]. Given that arginine metabolism via the arginase pathway leads to the production of polyamines and collagen, it is exciting to postulate that alternative activation of macrophages might contribute to the airway remodeling of asthma.

Arginase Inhibition and Allergic Inflammation

To elucidate the role of arginase in allergic inflammation, recent studies have explored arginase inhibition in animal models. These studies, involving different animal models and administration protocols, have yielded conflicting results, with one group reporting enhancement of inflammation [111], two groups reporting attenuation [44,74], and one group reporting no effect [140]. Mechanisms proposed to be important in the alteration of inflammatory status following arginase inhibition are the modulation of anti-inflammatory NO, pro-inflammatory peroxynitrite, and subsequent effects on the transcription factor, NFκ-B.

In a recent study, Ckless *et al.* investigated the effects of arginase inhibition in an ovalbumin sensitization and –challenge model of allergic airways inflammation employing female BALB/c mice [111]. Mice were sensitized to ovalbumin on days 0 and 7, and challenged for 30 min with aerosolized 1% ovalbumin or vehicle on days 14–16 [111]. The arginase inhibitor BEC was administered by oropharyngeal aspiration, two hours after the last challenge (40μl of 0.3mmol/L) and BAL and tissues were collected 46 hours later [111]. While BEC did not affect allergen-induced increases in differential cell counts or cytokine levels in BAL samples, the authors report significant enhancement of peribronchiolar and perivascular inflammation based on semi-quantitative scoring of histological sections [111]. Increases in both S-nitrosothiols and 3-nitrotyrosine were detected, without any apparent change in nitrite/nitrate levels [111]. The authors also report increased DNA binding of the NFκ-B transcription factor, resulting in augmentation of cDNA expression for proinflammatory cytokines, neutrophil attractant protein (KC) and macrophage inflammatory protein-3α (CCL20) [111]. The same group and others have previously shown that the activation of NFκ-B is redox-sensitive, activated by tyrosine nitration, but inhibited by S-nitrosothiols [141-143]. In lung epithelial cells the cytokine-induced NFκ-B activation was decreased following

arginase inhibition, with concomitantly increased concentrations of NO and S-nitrosothiols [144]. In another study, the cytokine-induced activation NFκ-B was decreased following arginase inhibition in lung epithelial cells, with concomitant increases in NO and s-nitrosothiols [144]. In addition the expression of KC and CCL20 in epithelial cells was attenuated by treatment with BEC [144]. Ckless *et al*. explain these apparent discrepancies by pointing to the possibility that increased NO produced following arginase inhibition may be converted into peroxynitrite [111]. While increased 3-nitrotyrosine was demonstrated in their study, S-nitrosothiols were also increased [111], making it unclear whether the balance lies in favour of reactive nitrogen species or anti-inflammatory S-nitrosothiols following arginase inhibition .

In another recently-published murine study, Bratt *et al*. demonstrated that administration of the arginase inhibitor, nor-NOHA (100 μg i.p), or vehicle, prior to each ovalbumin challenge reduced the total cells in BAL fluid by approximately 65% [74]. The authors postulated that arginase inhibition shunts L-arginine substrate towards the NOS pathway, partially blocking the allergen-induced inflammation through the anti-inflammatory effects of NO [74]. The same group recently demonstrated that increased expression of arginase in isolated airways correlate with levels of lung inflammation [110]. Interestingly, an increased inflammatory response to ovalbumin was observed in NOS2 knockout mice, supporting the anti-inflammatory effects of NO [74].

Recent studies in guinea pig models of allergen-induced airway inflammation also support the anti-inflammatory potential for arginase inhibition [44]. The arginase inhibitor ABH was administered by inhalation 30 minutes before and 8 hours after allergen challenge [44]. The allergen-induced increases in the numbers BAL cells, including eosinophils, macrophages and total inflammatory cell infiltration were all inhibited by ABH by approximately 50% [44]. As mentioned above, arginase inhibitors may reduce airway inflammation through an increase in anti-inflammatory NO production by cNOS as well as reduced NF-kB activation [44,94,144]. In addition, inhibition of arginase may also attenuate airway inflammation by reducing the formation of proinflammatory peroxynitrite via restoring the L-arginine availability to NOS2, thereby preventing the uncoupling of this enzyme, caused by allergen-induced upregulation of arginase [97]. Remarkably, the sensitivity of the airways to the inhaled allergen was also markedly reduced in the ABH-pretreated guinea pigs compared to saline-pretreated animals [44].

A recent study investigated the role of bone marrow cell-derived arginase 1 in the development of allergen-induced lung inflammation. To this aim, Niese *et al.* generated chimeric mice using irradiated CD45.1 congenic mice and bone marrow derived from arginase $1^{-/-}$ pups [140]. At 7–14 weeks post-irradiation, the authors employed two independent models of experimental asthma; the ovalbumin model and the *A. fumigatus* model [140]. In developing the chimeric mouse, the authors found that arginase 1 deficiency did not affect bone marrow reconstitution, immune cell development or adaptive immunity, as assessed by the production of allergen-specific antibodies [140]. Mice that received arginase $1^{-/-}$ bone marrow exhibited decreased arginase 1 expression by Northern blot and in situ hybridization following allergen challenge, compared to mice that received arginase $1^{+/-}$ bone marrow [140]. Additionally, the arginase 1-deficient chimeras did not exhibit significantly increased arginase activity following allergen challenge, indicating that bone marrow derived arginase 1 is the main contributor to increased arginase activity in experimental asthma [140]. In support, allergen-challenged arginase 2 knockout mice experience the same upregulation of lung arginase activity as wild type mice [140]. However, the ablation of arginase 1 from bone-marrow derived cells, or the complete knockout of arginase 2, did not affect basal nor allergen-induced inflammatory cell infiltration or differential counts in BAL [140]. These data suggest that arginase 2 and bone marrow-derived arginase 1 are not required for the development of lung inflammation in murine experimental asthma [140]. However, the results from the bone marrow cell derived arginase 1 deficient mice do not preclude the possibility that arginase 1 expressed in structural cells, such as the epithelial [43,99] or smooth muscle cells [39,43], may contribute to the development of allergic airways inflammation. The drastic reduction of arginase expression and activity did not induce an exacerbated inflammatory phenotype [140] as observed in the above-mentioned study using the arginase inhibitor BEC in mice [111].

By contrast, Pesce *et al*. recently generated arginase-1 floxed transgenic mice crossed with two independent macrophage-specific cre-recombinase expressing strains, resulting in two strains of mice in which arginase 1 was conditionally knocked out in the macrophages [139]. Using a model of *Schistosoma mansoni* infection, which typically occurs through Th2 cytokines, these investigators demonstrated that deletion of arginase 1 actually augmented the hepatic fibrosis and granulomatous inflammation in diseased animals [139]. Thus, in this model, arginase 1 expression in macrophages acts to suppress Th2-dependent fibrosis and inflammation [139].

Taken together, the role of arginase in airway inflammation in experimental asthma is not completely understood as both anti-inflammatory and pro-inflammatory effects of arginase inhibition have been observed, whereas knockdown of arginase 1 in bone marrow derived cells or total knockdown of arginase 2 does not affect lung inflammation. Further studies, using conditional knockdown of arginase 1 in structural cells, will be key in understanding the complex role of altered L-arginine metabolism in allergic inflammation. While many interesting advances in our understanding of the apparently divergent roles of L-arginine metabolism in allergic inflammation have been made recently in animal models, we must consider how these mechanisms may be manifest in human asthma, and how they may potentially be modulated for future therapies.

ARGINASE IN CHRONIC AIRWAY REMODELING

As previously described, chronic airway inflammation may induce airway remodeling. In particular, eosinophilia and secretion of TGF-β and IL-13 are considered to play an important role [145]. However, Holgate *et al.* suggested an important interactive role for the structural cells of the airways in the remodeling process as put forward by in the

epithelial mesenchymal trophic unit hypothesis [146-148]. Recent advances also implicate arginase in airway remodeling, through the production of L-ornithine and subsequent synthesis of polyamines and L-proline (**Fig 1**). Polyamines promote cell growth and proliferation, while L-proline is essential for collagen synthesis.

Lung fibrosis studies can also inform our understanding of the role of arginase in airway remodeling, as the mechanisms underlying increased collagen deposition in this disease likely exhibit some degree of overlap with those at work in asthma. Interestingly, the arginase activity in mouse lung tissue and fibroblasts was dose-dependently increased by the profibrotic factor TGF-β [149]. Moreover, levels of TGF-β, arginase activity and collagen content are all increased in rat lung allografts, with a strong correlation between arginase activity and collagen content [149]. Kitowska *et al.* recently explored the role of arginase in remodeling in lung fibrosis [150]. Increased expression of arginase 1, arginase 2 and collagen I, and a decrease in L-arginine availability, without alterations in protein arginine methyltransferases or CATs, were observed in mouse models of bleomycin-induced pulmonary fibrosis [101,151]. In addition, arginase 1 and 2 mRNA were induced by TGF-β1 in murine lung fibroblasts and inhibition of arginase by the nonspecific inhibitor NOHA attenuated TGF-β1-stimulated collagen deposition in a post-transcriptional manner [101].

Another group studying lung fibrosis recently interrogated the genetic pathways upregulated by TGF-β, using transgenic mice expressing human TGF-β1 under the control of the Clara cell 10-kD (CC10) promoter [152]. After treatment with doxycycline, these animals displayed upregulation of IL-13, infiltration of alternatively-activated macrophages and increased pulmonary fibrosis [152]. Importantly, arginase 1 and 2 expression and total arginase activity in BAL were also upregulated in the transgenic mice at day 14 post TGF-β1 induction [152]. These data support the hypothesis that arginase is induced by TGF-β as part of a pro-fibrotic genetic program, likely contributing via the metabolism of L-arginine to L-ornithine, leading to increased downstream production of proline and polyamines. These studies together provide a functional link between TGF-β1, arginase and collagen deposition in lung fibrosis. As TGF-β1 is also known to contribute to lung remodeling in asthma [153,154], these studies provide strong support towards arginase playing a key role.

In murine models of allergic airways inflammation there have also been recent developments implicating arginase in airway remodeling. Recently, treatment of mice in an ovalbumin model of allergic inflammation with mepacrine, an antimalarial drug, was shown to reduce the levels of IL-4, IL-13 and TGF-β1 as well as the expression and activity of arginases in the lung [155]. In this model, involving sensitization to ovalbumin on days 0, 7 and 14, followed by challenge with ovalbumin or vehicle beginning on day 21 for 12 consecutive days, Masson trichrome staining revealed sub-epithelial fibrosis, which was also attenuated by treatment with mepacrine[155]. However, mepacrine is also known to reduce Th2 cytokines and cysteinyl leukotrienes [155], therefore this study does not provide specific evidence that reduced arginase is the sole factor contributing to the reduction of remodeling in this model. To date no data is available regarding arginase inhibition with a specific inhibitor in a chronic model of allergic airways inflammation, but this study provides some encouraging findings. However, a recent study indicates that arginase 2 and bone marrow cell-derived arginase 1 are not important for allergen-induced fibrosis [140].

Increased arginase activity could also play a role in lung fibrosis by decreasing the NO production, since NO is antifibrotic. The importance of NO in regulating lung collagen content was supported in a guinea pig model of ongoing asthma. Thus, the allergen-induced collagen deposition around non-cartilaginous airways [156], but not in the alveolar septa [157] was further increased by chronic treatment with the NOS inhibitor L-NAME. Moreover, increased deposition of collagen in the lung has been observed in allergen-challenged $NOS2^{-/-}$ mice compared to allergen-challenged wild types [158].

Recently, novel studies have focused on the role of asymmetric dimethylarginine (ADMA) in respiratory function and disease. ADMA is an endogenous competitive inhibitor of NOS, derived from the breakdown of proteins containing L-arginine residues previously methylated by protein-arginine methyltranserases (PRMTs; **Fig 1**) [159,160]. The role of ADMA in the regulation of the L-arginine/NO pathway *in vivo* and airway remodeling is just beginning to be elucidated. Wells *et al*. infused saline or ADMA (30–90 mg/kg/day) via implanted osmotic minipumps in naïve Balb/c mice for 2 weeks [161]. Increased lung hydroxyproline content – as a marker for lung fibrosis - and collagen staining on histological sections were observed in ADMA-treated mice compared with saline-treated controls with concomitant higher arginase activity, decreased nitrite concentrations and increased urea in lung homogenates [161]. Interestingly, ADMA treatment did not affect IL-13 or arginase expression [161]. Finally, it was shown that ADMA induced an increase in arginase *activity* and collagen fiber formation in primary mouse lung fibroblast cultures, and that this was preventable through addition of NOHA [161]. In addition, treatment with the NOS inhibitor L-NAME has been shown to further increase the IL-4-induced production of TGF-β by lung fibroblasts [162].

Another characteristic of airway remodeling is increased smooth muscle mass. In the vasculature, arginase has been shown to play a role in smooth muscle proliferation [163,164]. Elevated production of polyamines from L-ornithine as well as decreased synthesis of NO due to increased arginase activity could therefore contribute to the process of airway smooth muscle thickening . The synthesis of the polyamines (putrescine, spermidine and spermine) is initiated by ornithine decarboxylase (ODC), which converts L-ornithine into putrescine [30]. Polyamines are involved in cell proliferation [3,30,55,80,165] and induce the expression of genes involved in cell proliferation by promoting histone acetyltransferase activity and chromatin hyperacetylation [166]. Studies in the vasculature have shown that the expression/activation of arginase and ODC can be induced by growth factors, leading to increased polyamine levels [167-171] and this activation of ODC has also been observed in the airway smooth muscle [172]. Importantly, levels of polyamines in lungs of allergen-challenged mice [99] and in

serum of asthmatic patients are increased [173]. The potential importance of arginase in this context is illustrated by the observation that arginase activity in the airways and lungs are increased in animal models of chronic allergic asthma [43,72].

In contrast to polyamines, NO inhibits mitogen-induced proliferation of cultured airway smooth muscle cells from human [174-176] and guinea pig [177] origins, by inhibiting the G_1 phase and the S phase of the cell cycle [175] This inhibitory effect of NO on airway smooth muscle proliferation is mediated by the activation of soluble guanylyl cyclase and subsequent cGMP production [174,175,177], but also involves cGMP-independent inhibition of ribonucleotide reductase [175]. The exact downstream mechanisms contributing to NO-mediated inhibition of airway smooth muscle cell proliferations are currently unknown, but studies in vascular smooth muscle cells have shown that NO represses cell cycle promoting genes and induces inhibitors of the cell cycle [178] and inhibits PDGF-induced activation of protein kinase B [179]. In addition, NO inhibits the activation of ODC via S-nitrosylation of the enzyme under normal conditions [180].

Bergeron *et al*. examined L-arginine related proteins by immunostaining in asthmatic airways [39]. This study revealed increased arginase expression in asthmatics who smoke, localized to epithelia and the peri-bronchiolar region. This study suggests a role for arginase in injury and repair initiated by cigarette smoke insult [39]. It also demonstrates arginase expression in steroid-naïve asthmatics in anatomical proximity to remodeling features.

Conclusively, these findings suggest that increased arginase activity in chronic asthma could also contribute to the airway smooth muscle thickening via an increased production of polyamines from L-ornithine and via a reduced formation of NO. Studies using arginase inhibitors in animal models are warranted to elucidate the exact role of arginase in airway remodeling in chronic asthma.

CELLULAR AND SUB-CELLULAR LOCALIZATION

A key level at which NOS and arginase can compete for substrate is through cellular and subcellular localization throughout the airways. Co-expression of arginase and NOS has been demonstrated in epithelial cells of the airways. The epithelial cells of the large airways are known to undergo significant changes in asthma, and their importance in AHR is beginning to be appreciated [181,182]. Damage and shedding of the epithelia has been observed in asthmatics and may contribute to the loss of bronchoprotection by cNOS-derived NO [88]. Increased arginase 1 has been shown in asthmatic airway epithelial cells in bronchoscopy samples [39,99]. Recent human data and murine models of allergic airways inflammation suggest expression in the apical layer of airway epithelial cells [43,150].

Importantly, concomitant expression of NOS1 and arginase in airway epithelial cells supports the potential for arginase overexpression to reduce the local bioavailability of L-arginine for NOS1 in these cell types [102,111]. NOS1 has been demonstrated by ourselves and others to localize primarily to airway epithelial and smooth muscle cells [43,182]. As noted above, previous studies in guinea pigs have suggested that arginase activity can affect airway tone through increased competition with NOS1 in both homeostasis and under conditions of allergic inflammation [17,42,86,98]. Thus, arginase and NOS1 may be in competition for intracellular L-arginine pools within the same cells and under conditions of allergic inflammation this competition may be exacerbated by increased arginase 1 expression.

In addition to whether or not specific cell types co-express the arginase/NOS isozymes, sub-cellular localization of L-arginine-related enzymes may be critical to understanding the outcome of dysregulation of proteins related to L-arginine metabolism. NOS1 contains a PDZ domain that participates in the formation of active dimers, and interactions with other proteins [183,184]. NOS1 has been localized to the cytosol, neuronal synapses and the plasma membrane [185,186]. In contrast, NOS2 has a relatively wide-ranging distribution in various cell types, including the cytosol, perinuclear space, plasma membrane and mitochondria [187-189]. NOS3 has been demonstrated at the plasma membrane, where it has complex intractions with caveolin and CAT1 [185,190-192]. Similar to NOS2, NOS3 has also been reported to be present in mitochondria [189]. Meanwhile, arginase 1 is localized primarily in the cytosol, while arginase 2 is confined to the mitochondria. Both arginase 1 and 2 have been implicated in regulating NOS1 activity through the depletion of intracellular L-arginine [193,194]. In cultured endothelial cells arginase 2 has been demonstrated to regulate the activity of NOS3, and this regulation has been shown to take place within non-freely exchangeable L-arginine pools [195,196]. Furthermore, over-expression of arginase 1 has been demonstrated to result in impaired NO production by NOS1 in cultured 293 embryonic kidney cells stably transfected with NOS1 [194]. Ckless *et al*. investigated the colocalization of arginase 1 with NOS isoforms in C10 murine alveolar type II epithelial cells [144]. Through immunoprecipitation it was demonstrated that arginase 1 colocalized with NOS3, but not NOS1 or NOS2 in this cell type [144].

The subcellular localization of the arginase isoenzymes may also have implications in the downstream fates of the products of arginase and NOS, L-ornithine, and L-citrulline, respectively. The localization of ornithine decarboxylase (ODC) in the cytosol may preferentially steer cytosolic L-ornithine towards the production of polyamines, which are important in cell proliferation. Ornithine amino transferase (OAT) is localized to the mitochondria, potentially directing the L-ornithine produced by arginase 2 towards the production of proline and collagen synthesis [197]. However, ,transport of L-ornithine across the mitochondrial membrane is known to occur [30]. Furthermore, recycling of L-citrulline to L-arginine by ASS and ASL may be important in regulating L-arginine availability for the NOS isoenzymes. ASS produces argininosuccinate from L-citrulline and aspartate as part of the L-citrulline-NO cycle, whereas ASL cleaves argininosuccinate into L-arginine and fumarate [30]. Both ASS and ASL are localized in the cytoplasm, indicating that the full recycling of L-citrulline to L-arginine may take place within that cellular compartment [198]. Future investigations of the role(s) of L-arginine-related proteins/pathways in the pathogenesis of disease will need to consider the potential impact of sub-cellular localization of

competing NOS and arginase isozymes and the pathways of downstream metabolism of their respective products.

In a recent paper examining the effectiveness of inhaled steroids on preventing remodeling, Kai *et al.* recently investigated collagen synthesis [199]. The authors measured sputum levels of procollagen type I C-terminal peptide (PICP), a marker of ongoing collagen synthesis, in 25 normal volunteers and 25 steroid-naive asthmatics, who immediately began steroid treatment and were followed up one month later [199]. Asthmatics exhibited increased PICP, relative to controls, consistent with a previous study by the same authors. While treatment with inhaled corticosteroids improved the FEV1, and decreased sputum eosinophil counts, TGF-β^+ cells and sputum PICP concentrations, treatment did not normalize PICP concentrations to the level of controls, suggesting ongoing airway remodeling in steroid-treated asthmatics [200].

The effect of steroids on arginase expression in human asthma is currently unclear. There is some evidence that increased arginase expression can be induced by steroids, such as in the digestive tract of piglets treated with glucocorticoids [201]. Dexamethasone has also been found to increase arginase activity when administered to rescue mice from endotoxemia [202]. Whereas, in rat airway fibroblasts, dexamethasone has been reported to inhibit the IL-4/IL-13 induced upregulation of arginase [38]. However, dexamethasone was also reported to have no effect on endotoxin/TNFα-induced upregulation of arginase in bovine pulmonary arterial endothelial cells [203]. More studies on the effects of corticosteroid treatment on arginase expression in asthma are needed, but a recent study by Lara *et al.*, provides some information. In this study of severe asthmatics, moderate asthmatics and healthy controls, the severe asthmatics exhibited increased serum arginase activity, which was related inversely to airflow, despite the fact that 95.2% of these patients were being treated with inhaled corticosteroids, and 38.1% with systemic corticosteriods [114]. Thus, there is significant potential for new therapies to target arginase, as current treatments do not appear to attenuate the activity of this pathway or prevent remodeling.

FUTURE DIRECTIONS

Increased arginase activity importantly contributes to the development of key processes of allergic asthma, such as AHR, airway inflammation and – possibly – remodeling. Although some conflicting results have been reported, most studies investigating the effect *in vivo* inhibition of arginase demonstrate a beneficial effect of arginase inhibitors on AHR and airway inflammation. Therefore, arginase inhibitors may have therapeutic potential in the treatment of allergic asthma. Future experiments are warranted to elucidate the specific role the two arginase isozymes in disease pathogenesis and in homeostasis. To this aim, further investigation of new, more selective inhibitors and methods of administration that will minimize non-specific effects (i.e., inhibition of arginase in extrapulmonary tissues) as well as the use of conditional knockout animals are needed.

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